REMARKS

Claims 8, 11-14, 33-38, 40, 41, 49-56, and 58-60 having been cancelled, claims 1, 4-6, 9, 10, 15-17, 25, 29, 31, 32, 39, 42-48, 57, and 61 having been amended, and claims 62-74 having been added, the claims presently pending in the above-identified application are claims 1-7, 9, 10, 15-32, 39, 42-48, 57, and 61-74.

Amendments to the Claims

The claim amendments find support throughout the specification. For example, the recitation of hybridization conditions in claims 1, 31, 32, 48, and 61 is supported by the specification at, for example, page 12, lines 1-15. The recitation in claims 1 and 48, and in new claims 63 and 68, of the absence of phenol or chloroform is supported by the specification at, for example, page 13, lines 15 and 16. The recitation of a vertebrate cell in claims 1 and 48 is supported by the specification at page 2, line 27. The recitation of specific attenuation of gene expression in claims 1 and 48 is supported by the specification at, for example, page 2, line 30. The recitation in claims 15 and 16 of an RNA sequence complementary to a portion or region of the target gene is supported by the specification at, for example, page 12, lines 16-24.

Claims 4-6, 17, and 25 were amended to clarify the wording the Examiner found indefinite in the Office Action mailed February 12, 2001, in the parent application, U.S. Serial No. 09/493,301. Claims 9, 10, 39, 42-47, and 57 were amended to correct claim dependencies. Claim 29 is amended to refer to an appropriate antecedent.

New claims 62-74 also find support throughout the specification, for example, as follows, claims 62-65, pages 2, 3, 12, and 13; claim 66, page 3, 4, and 12; claim 67, pages 12 and 13; claims 68-71, pages 4 and 10; claims 72 and 73, pages 3 and 4; and claim 74, pages 4 and 14.

Amendments to the Specification

Example III (paragraph beginning at page 36, line 10) and Fig. 13 (including the legend at page 8, lines 3-4) in the application as originally filed mistakenly identified the mammalian cells used in cell culture as murine NIH/3T3 cells. As stated in the Supplemental Declaration Under 37 C.F.R. §1.132 of Dr. Yin-Xiong Li, submitted herewith, the cells actually used in this

working example were rat ROS cells. Support for the recitation of "rat cell" in the amended specification is found, for example, at page 10, lines 17-23 of the specification.

Substitute Drawing

As indicated in the Supplemental Declaration Under 37 C.F.R. §1.132 of Dr. Li, Figure 13 contains data generated using dsRNA transfected into rat ROS cells, not murine NIH/3T3 cells. The cells were mistakenly indicated as NIH3T3 cells in Fig. 13 as originally filed. Accordingly, the title of the drawing is changed to reflect data generated in "rat" cells, since the cells actually producing the data were rat ROS cells. Support for the recitation of rat cells is found in the specification at, for example, page 10, lines 17-23. A copy of the originally filed drawing, marked in red to show the changes, is submitted herewith (Exhibit A), as is a copy of the substitute drawing. Approval and entry of the substitute drawing is respectfully requested.

Rejection Under 35 U.S.C. §112, first paragraph, in the Parent Application, U.S. Serial No. 09/493,301

If and to the extent the rejection under 35 U.S.C. §112, first paragraph, that was made in the parent application in the Office Action mailed February 12, 2001, is applied to the claims as presently pending, it is respectfully traversed. The Examiner acknowledges that the specification is enabling for methods of attenuating the expression of the specific disclosed target genes in zebrafish, avian neural crest tissue explants, and murine NIH/3T3 cells. However, the Examiner asserts that the specification is not enabling for methods of attenuating the expression of any gene in any vertebrate cell *in vivo* or *in vitro*. In particular, the Examiner states that the specification does not provide sufficient guidance as to which genes should be targeted and how to administer an effective amount of the dsRNA to attenuate expression of the target gene. Applicants respectfully disagree.

Applicants assert that the pending claims are fully enabled by the specification. A key aspect of Applicants' dsRNA-mediated gene silencing methods is that they can be generally applied to silence any target gene of interest. As stated on page 10, lines 8-16, of the specification:

Targeted gene

Any gene being expressed in a cell can be targeted. A gene that is expressed in the cell is one that is transcribed to yield an mRNA and, optionally, a protein. The targeted gene can be chromosomal (i.e., genomic) or extrachromosomal. It may be endogenous to the cell, or it may be a foreign gene (i.e., a transgene). The foreign gene can be integrated into the host genome, or it may be present on an extrachromosomal genetic construct such as a plasmid or a cosmid. The targeted gene can also be derived from a pathogen, such as a virus, bacterium, fungus or protozoan, which is capable of infecting an organism or cell.

The specification further teaches:

> In medical applications, the target gene can be an endogenous gene of the organism, or can be the gene of a pathogen. For example, dsRNA may be introduced into a cancerous cell or tumor, and thereby inhibit expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. An exemplary list of potential target genes, including developmental genes, oncogenes, and enzymes, and a list of cancers that can be treated according to the present invention can be found in WO 99/32619 (Fire et al., published 1 July 1999). A candidate target gene derived from a pathogen might, for example, cause immunosuppression of the host or be involved in replication of the pathogen, transmission of the pathogen, or maintenance of the infection.
>
> The method of the invention can also be used to regulate the expression of an exogenous gene or "transgene" that has been introduced into a host plant or animal. For example, a transgene that is present in the genome of a cell as a result of genomic integration of the viral delivery construct can be regulated using dsRNA according to the invention. (Page 16, lines 8-22).

Additionally, the specification states that genes of unknown function can be silenced to determine their physiological roles:

> The method of the present invention is also useful to identify and characterize gene function in an organism. In this "functional genomics" approach, dsRNA is targeted to a gene of previously unknown function, and the resultant change in phenotype is observed and, optionally, quantified. This approach is useful to identify potential targets for pharmaceutics, to promote understanding normal and pathological events associated with development, to determine signaling pathways responsible for postnatal development and aging, and the like. For example, dsRNA can be designed to target a partial sequence of an expressed sequence tag (EST). Functional alterations in growth, development,

> metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product. As another example, dsRNA targeted to new genes found by genomic sequencing programs or other "data mining" of genomic data can be used to understand the physiological roles of these new genes. The ease with which dsRNA can be introduced into an intact cell or organism containing the target gene allows the present invention to be used in high throughput screening (HTS) applications. For example, dsRNA can be produced by an amplification reaction using primers flanking the inserts of any cDNA or genomic DNA gene library derived from the target cell or organism. (Page 17, lines 5-23).

Based on these teachings in the Applicants' specification, one skilled in the art would appreciate that the methods of the present invention can be used to silence numerous endogenous genes (e.g., disease-related genes), pathogen genes, and novel genes of unknown function. In particular, dsRNA molecules can be designed to silence any target gene of interest for which the polynucleotide sequence or a region of the polynucleotide sequence is known. The structural characteristics of dsRNA molecules that silence a gene of interest are sufficiently described in Applicants' specification by their sequence identity to the target gene, by their preferred lengths, and/or by hybridization conditions (see, for example, Applicant's specification, page 12, lines 13-14) to enable one skilled in the art to design dsRNA molecules for use in the claimed methods (see, for example, pages 11 and 12 of the specification). As stated on page 11, lines 19-31, of the specification:

> The nucleotide sequence of the dsRNA is defined by the nucleotide sequence of its targeted gene. The dsRNA contains a nucleotide sequence that is essentially identical to at least a portion of the target gene; preferably the dsRNA contains a nucleotide sequence that is completely identical to at least a portion of the target gene. It should be understood that in comparing an RNA sequence to a DNA sequence, an "identical" RNA sequence will contain ribonucleotides where the DNA sequence contains deoxyribonucleotides, and further that the RNA sequence will contain a uracil at positions where the DNA sequence contains thymidine. More preferably, the dsRNA that is completely identical to at least a portion of the target gene does not contain any additional nucleotides. The portion of the target gene to which the dsRNA sequence is essentially or

> completely identical is preferably a sequence that is unique to the genome of the cell into which the dsRNA is to be introduced.

Alternatively, dsRNA molecules can be used to identify genes of unknown sequence. For example, dsRNA molecules can be synthesized using cDNA or genomic DNA libraries and used in high throughput screening methods to identify genes that produce a desired phenotype when silenced (see, for example, page 17 of the specification).

Moreover, the specification describes several routine methods for synthesizing dsRNA molecules *in vitro* and for synthesizing vectors encoding dsRNA molecules to produce the desired dsRNA molecule *in vivo* (see, for example, pages 4, 5, 11-15, 21 and 22 of the specification). At page 15, lines 4-20, the specification also teaches the following exemplary doses of dsRNA for gene silencing in vertebrate cells:

> The amount of dsRNA administered to a cell, tissue, or organism depends on the nature of the cell, tissue, or organism, the nature of the target gene, and the nature of the dsRNA, and can readily be optimized to obtain the desired level of gene inhibition. To attenuate gene expression in a single cell embryo, for example, at least about 0.8×10^6 molecules of dsRNA are injected; more preferably, at least about 20×10^6 molecules of dsRNA are injected; most preferably, at least about 50×10^6 molecules of dsRNA are injected. The amount of dsRNA injected into a single cell embryo is, however, preferably at most about 1000×10^6 molecules; more preferably, it is at most about 500×10^6 molecules, most preferably, at most about 100×10^6 molecules. In the case of administration of dsRNA to a cell culture or to cells in tissue, by methods other than injection, for example by soaking, electroporation, or lipid-mediated transfection, the cells are preferably exposed to similar levels of dsRNA in the medium. For example, 8- 10 μL of cell culture or tissue can be contacted with about 20×10^6 to about 2000×10^6 molecules of dsRNA, more preferably about 100×10^6 to about 500×10^6 molecules of dsRNA, for effective attenuation of gene expression.

As noted by the Examiner, the specification also discloses a variety of routes of administration for introducing dsRNA into vertebrate cells (see, for example, pages 14 and 15 of the specification). Alternatively, a vector encoding dsRNA can be administered to vertebrate cells (see, for example, page 15 of the specification). Various standard methods for introducing vectors into vertebrate cells were known to skilled artisans at the time the application was filed.

The specification further provides numerous routine assays that can be used to confirm the ability of a particular dsRNA molecule to silence a target gene or to screen for a dsRNA molecule that silences the target gene (as disclosed, for example, on pages 8-10). For example, as stated on page 8, line 25 through page 9, line 13 of the specification:

> Inhibition of gene function is evidenced by a reduction or elimination, in the cell, of the activity associated with the protein encoded by the gene. Whether and to what extent gene function is inhibited can be determined using methods known in the art. For example, in many cases inhibition of gene function leads to a change in phenotype which is revealed by examination of the outward properties of the cell or organism or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP),beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

In summary, Applicants clearly describe, in the specification, how to design a dsRNA molecule to silence a target gene of interest, how to administer an appropriate dose of the dsRNA molecule or a vector encoding the dsRNA molecule to a vertebrate cell, and how to assay the cell for inhibition of the target gene. If desired, multiple dsRNA molecules or multiple doses of dsRNA can be rapidly tested to optimize the inhibition of the target gene. In addition, the specification provides numerous working examples of successful dsRNA-mediated gene silencing in vertebrate cells using micoinjected or transfected dsRNA (Examples I and III). Due to the ease in which dsRNA can be introduced into vertebrate cells, a skilled artisan would appreciate that undue experimentation is not necessary to carry out the claimed methods. As *In re Vaeck* states:

> [t]he first paragraph of 35 US.C. § 112 requires, *inter alia*, that the specification of a patent enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without "undue experimentation" . . . That *some* experimentation may be required is not fatal; the issue is whether the amount of experimentation required is "undue." See 20 USPQ2d 1438, 1444 (Fed. Cir. 1991).

In response to the assertion by the Examiner that post-filing references Oates *et al.* (Dev. Biol., 224:20-28, 2000) and Zhao *et al.* (Dev. Biol. 229:215-223, 2001) report non-specific effects of dsRNA in zebrafish embryos, Applicants note that the specification discloses *specific inhibition* of *four different genes* in zebrafish embryos. The phenotypes that were generated for each gene were substantially different from one another and were specifically and predictably related to the gene that was targeted (page 34, line 32 to page 35, line 9, of the specification). Applicants are well aware that others have observed nonspecific effects in similar experiments and addressed the issues of toxicity and nonspecificity in the specification at page 34, line 14, bridging to page 35, line 9:

> Finally, it is known that certain types of double-stranded RNA, such as mismatched or polyI/polyC RNA, can be toxic at high concentrations in eukaryotic animals (M. Kumar et al., *Microbiol. Mol. Biol. Rev.* 62, 1415-1434 (1998)). Although double-stranded RNA can induce interferon-α/β in non-immune cells, this toxicity is primarily due to an immune system response mediated through interferon production in response to viral infections. Immune system or interferon-α/β -mediated toxicity is very unlikely to play any role in generating the phenotypes we have observed. First, the phenotypes that we have generated can be observed in 24 hour embryos, long before the zebrafish immune system has been established. The thymus primordium appears in the zebrafish at approximately 54 hours, but does not enlarge significantly until 30 hours later. Rag1 and Rag2 expression cannot be detected until day 4, indicating a lack of mature T cells in the zebrafish until that time. Second, the amount of double-stranded RNA that was used to generate the phenotypes is much less than is necessary to cause this interferon-mediated cell toxicity (M. Kumar et al., *Microbiol. Mol. Biol. Rev.* 62, 1415-1434 (1998)). We have also found that polyI/polyC RNA can be toxic both in cultured 3T3 cells and in microinjected embryos. However, none of the ten double-stranded RNAs that we have so far examined elicit a toxic effect *in vitro* or *in vivo*. Third, the phenotypes that have been generated for each gene under study differ substantially from one another

> and are specifically related to the gene that was targeted. Finally, injection of control double-stranded RNA at the same concentrations does not cause a detectable deviation from the wild type expression levels or phenotype.
>
> In summary, these results show that double-stranded RNA can efficiently disrupt gene activity in zebrafish. This inhibitory activity appears to be specific to the targeted gene. Non-specific double-stranded RNA had no apparent phenotypic effect. We have also shown that multiple genes can be simultaneously targeted.

The Declaration Under 37 C.F.R. § 1.132 of Dr. Yin-Xiong Li, submitted herewith, states that any non-specific effects reported by Oates or Zhao can be explained by differences between their methods and the methods taught in Applicants' specification. For example, both Oates and Zhao use dsRNA formed from single-stranded RNA that has been purified by phenol/chloroform extraction (see, for example, page 216, first column of Zhao and page 21, first column of Oates). As stated in the Li Declaration, phenol is toxic to zebrafish embryos and causes concentration-dependent, adverse effects in zebrafish. Thus, any non-specific effects observed by Oates or Zhao are thought to be the result of toxic effects from residual phenol and/or chloroform in the mixture injected into the zebrafish embryos. Declaration under 37 C.F.R. § 1.132 of Dr. Yin-Xiong Li.

In contrast, Applicants' specification teaches the desirability of using dsRNA that has been formed from single stranded RNA purified without the use of phenol or chloroform (see, for example, the specification at page 13, lines 15 and 16; and page 21, line 12, bridging to page 22, line 8, wherein dsRNA is prepared without the use of phenol or chloroform). Thus, the specification enables the present methods for *specifically* silencing target genes in vertebrate cells. Moreover, the genes are silenced without toxic effects.

In response to the assertion that Wianny *et al.* (Nature Cell Biology 2:70-75, 2000) mentions the possibility of difficulties in using dsRNA to silence mouse genes during later stages of development, Applicants note that Wianny does not report any difficulties in silencing genes in murine cells. In contrast, Wianny states that "we have shown here that the injection of a dsRNA is specific to the corresponding gene; it does not cause a general translational arrest, because embryos continue to develop and we see no signs of cell death" (page 73, column 2, second paragraph).

As discussed in the attached Declaration Under 37 C.F.R. §1.132 of Dr. Satishchandran, submitted herewith, the post-filing reference (Ui-Tei *et al.*, FEBS Letters 479:79-82, 2000) (a copy of which is submitted herewith) demonstrates that dsRNA or a plasmid encoding dsRNA can be used to specifically silence a gene in mammalian Chinese hamster cells. This reference further supports the ability of the presently claimed methods to specifically silence vertebrate (e.g., mammalian) genes.

Additionally, the Satishchandran Declaration demonstrates the ability of a vector encoding dsRNA to specifically silence the prostate-specific antigen (PSA) gene in human rhabdomyosarcoma cells containing a PSA expression plasmid (U.S. Ser. No. 10/062,707, filed January 31, 2002, published Sept. 19, 2002 as US 2002/0132257 A1). Transfection of the vector encoding the dsRNA was mediated by lipofectamine (Gibco-BRL) according to the manufacturer's instructions. A greater than 95% reduction in PSA expression was seen in the cells expressing the PSA dsRNA. The inhibition was seen within two days after transfection and continued up until the last time point taken (one month later), at which point PSA levels were beginning to decline in the untreated cells and the experiment was terminated. The down-regulation was sequence specific: only PSA derived dsRNA, and not control HSV-2 derived dsRNA, induced down-regulation of PSA. Additionally, there was no toxicity associated with the cytoplasmic expression of the dsRNA.

In summary, the specification teaches the generation of dsRNA, the introduction of dsRNA into vertebrate cells, and the identification of cells having the desired level of target gene inhibition. The Applicants' specification, the post-filing Ui-Tei reference, and the Satishchandran Declaration demonstrate several working examples of successful gene silencing in vertebrate cells. Based on the above evidence, it is respectfully submitted that the present claims are fully enabled by the specification.

CONCLUSION

Applicants submit that this application is now in condition for allowance. The Examiner is invited to contact Applicants' Representatives at the below-listed telephone number, if there are any questions regarding this Preliminary Amendment or if prosecution of this application may be in any way expedited or assisted thereby.

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APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS INCLUDING NOTATIONS TO INDICATE CHANGES MADE
Serial No.: 10/038,984
Docket No.: 275.0003 0102

Amendments to the following are indicated by underlining what has been added and bracketing what has been deleted. Additionally, all amendments have been shaded.

In the Specification

The paragraph beginning at page 8, lines 3-4, has been amended as follows:

Figure 13 shows the effect of GFP double-stranded RNA injection on transient expression of GFP in [murine] rat cell culture.

The paragraphs beginning at page 36, line 10 through page 37, line 8, has been amended as follows:

Double-stranded GFP RNA was prepared as described in Example I. [Murine NIH/3T3] Rat cells were transfected with pEGFP-N1 and double stranded GFP RNA using a standard transfection procedure. First, [murine NIH/3T3] cells (~2x 10^8 per well) were seeded in a six-well tissue culture plate in 2 ml of DMEM with 10% FBS. The cells were then incubated at 37°C in a CO_2 incubator until they were about 70-80 % confluent (i.e., 18-24 hours).

In the Claims

A marked-up version of claims pending after amendment is presented below. For convenience, all pending claims are shown.

1. (Amended) A method for attenuating the expression of a target gene in a vertebrate cell comprising [introducing into the cell] supplying the cell with a double stranded RNA in an amount sufficient to specifically attenuate expression of the target gene, wherein one of the strands of the double stranded RNA is capable of hybridizing to the target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C, and provided that, when the double

stranded RNA is supplied to the cell by delivery to the cell of double stranded RNA, the double stranded RNA is formed from single-stranded RNA that is purified in the absence of phenol or chloroform [the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene].

2. The method of claim 1 wherein the target gene is an endogenous gene.

3. The method of claim 1 wherein the target gene is a foreign gene.

4. (Amended) The method of claim 1 wherein the target [targeted] gene is a chromosomal gene.

5. (Amended) The method of claim 1 wherein the target [targeted] gene is an extrachromosomal gene.

6. (Amended) The method of claim 1 wherein the target [targeted] gene is [derived] from a pathogen capable of infecting the cell.

7. The method of claim 6 wherein the pathogen is selected from the group consisting of a virus, bacterium, fungus or protozoan.

9. (Amended) The method of claim 1 [8] wherein the vertebrate cell is a fish cell.

10. (Amended) The method of claim 1 [8] wherein the vertebrate cell is a mammalian cell.

15. (Amended) The method of claim 1 wherein the double stranded RNA comprises a nucleotide sequence that is complementary [completely identical] to the nucleotide sequence of at least a portion of the target gene.

16. (Amended) The method of claim 1 [in which the essentially identical nucleotide sequence is at least] wherein the double stranded RNA comprises a nucleotide sequence that is complementary to a region of at least 50 bases [in length] of the target gene.

17. (Amended) The method of claim 1 wherein the double stranded RNA is supplied [administered] in an amount sufficient to completely inhibit expression of the target gene.

18. (Amended) The method of claim 1 in which the double stranded RNA comprises [one] a single strand which is self-complementary.

19. The method of claim 1 in which the double stranded RNA comprises two separate complementary strands.

20. The method of claim 1 wherein the cell is an embryo.

21. The method of claim 20 wherein the embryo is a fish embryo.

22. (Amended) The method of claim 20 wherein the embryo is supplied with the double stranded RNA [is introduced into the embryo] using microinjection.

23. The method of claim 1 wherein the cell is present in a cell culture, a tissue, an organ, or an organism.

24. (Amended) The method of claim 23 wherein the cell is present in an organism, and the cell is supplied with the double stranded RNA [is introduced] by introducing double stranded RNA into a body cavity or interstitial space of the organism.

25. (Amended) The method of claim 23 wherein the cell is present in an organism, and wherein the cell is supplied with the double stranded RNA [delivered to] by delivering double stranded RNA to the organism via oral, topical, parenteral, vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration.

26. (Amended) The method of claim 23 wherein the cell is present in a cell culture or a tissue explant, and wherein [the introduction of the double stranded RNA into] the cell is supplied with the double stranded RNA by [comprises] incubating the cell culture or tissue explant in a solution comprising the double stranded RNA.

27. (Amended) The method of claim 1 wherein supplying the double stranded RNA to the cell comprises delivering double-stranded RNA to the cell, and wherein the double stranded RNA is treated with RNAse prior to [its introduction into] delivery to the cell.

28. (Amended) The method of claim 1 wherein supplying the double stranded RNA to the cell comprises delivering double stranded RNA to the cell, the method further comprising, prior to [introducing] delivering the double stranded RNA [into] to the cell, annealing two complementary single stranded RNAs to yield the double stranded RNA.

29. (Amended) The method of claim [1] 28 wherein the [complementary] single stranded RNAs are annealed in the presence of potassium chloride.

30. The method of claim 1 wherein the function of the target gene is unknown.

31. (Amended) The method of claim 1 further comprising introducing into the cell a second double stranded RNA in an amount sufficient to attenuate expression of a second target gene, wherein one of the strands of the second double stranded RNA is capable of hybridizing to the second target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C

[the second double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the second target gene].

32. (Amended) The method of claim 1 comprising introducing into the cell multiple double stranded RNAs in an amount sufficient to attenuate expression of multiple target genes, wherein one strand of each double stranded RNA is capable of hybridizing to the corresponding target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C [double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of a target gene].

39. (Amended) The method of claim 23 [37] further comprising identifying a phenotypic change in the cell culture associated with attenuated expression of the target gene.

42. (Amended) The method of claim 66 [41] wherein the [tissue explant exhibiting attenuated expression of the target gene is implanted back into the organism] donor and the recipient are the same.

43. (Amended) The method of claim 66 [41] wherein the [tissue explant exhibiting attenuated expression of the target gene is implanted into a second organism] donor and the recipient are different.

44. (Amended) The method of claim 66 [41] wherein the tissue is fetal tissue.

45. (Amended) The method of claim 66 [41] wherein the donor and recipient organisms are vertebrates [organism is a vertebrate].

46. (Amended) The method of claim 66 [41] further comprising identifying a phenotypic change in the tissue [explant] associated with attenuated expression of the target gene.

47. (Amended) The method of claim 66 [41] wherein expression of the target gene [in the organism] is completely inhibited.

48. (Amended) A method for attenuating the expression of a target gene in a vertebrate cell comprising:

annealing two complementary single stranded RNAs in the presence of potassium chloride to yield double stranded RNA;

contacting the double stranded RNA with RNAse to purify the double stranded RNA by removing single stranded RNA; and

introducing the purified double stranded RNA into the cell in an amount sufficient to specifically attenuate expression of the target gene;

wherein one of the strands of the double stranded RNA is capable of hybridizing to the target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C, and wherein the double stranded RNA is formed from single-stranded RNA that is purified in the absence of phenol or chloroform [the double stranded RNA comprises a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene].

57. (Amended) The method of claim 66 [56] wherein the transplant tissue is hepatocytes.

61. (Amended) A kit comprising reagents for attenuating the expression of a target gene in a cell, the kit comprising:

a DNA template comprising two different promoters selected from the group consisting of a T7 promoter, a T3 promoter and an SP6 promoter, each promoter operably linked to a nucleotide sequence, such that two complementary single stranded RNAs are capable of being transcribed from the DNA template [, and wherein the complementary single stranded RNAs comprise a nucleotide sequence that is essentially identical to the nucleotide sequence of at least a portion of the target gene];

a plurality of primers for amplification of the nucleotide sequence;

nucleotide triphosphates for forming RNA;

at least two RNA polymerases, each capable of binding to a promoter on the DNA template and causing transcription of the nucleotide sequence to which the promoter is operably linked;

a purification column for purifying single stranded RNA;

buffer for annealing single stranded RNAs to yield double stranded RNA; and

RNAse A or RNAse T for purifying double stranded RNA;

wherein one of the strands of the double stranded RNA is capable of hybridizing to the target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C.

62. (New) The method of claim 1 wherein one of the strands of the double stranded RNA is capable of hybridizing to the target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 70°C.

63. (New) A method for attenuating the expression of a target gene in a vertebrate cell comprising delivering a double stranded RNA to the cell in an amount sufficient to specifically attenuate expression of the target gene, wherein the double stranded RNA comprises a nucleotide sequence that is complementary to a region of at least 25 nucleotides of the target gene, and wherein the double stranded RNA is formed from single-stranded RNA that is purified in the absence of phenol or chloroform.

64. (New) A method for attenuating the expression of two or more target genes in a vertebrate cell, the method comprising supplying the cell with two or more double stranded RNAs in an amount sufficient to specifically attenuate expression of the target genes, wherein one strand of each double stranded RNA is capable of hybridizing to the corresponding target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C.

65. (New) A method for attenuating the expression of two or more target genes in a vertebrate cell, the method comprising supplying the cell with two or more double stranded RNAs in an amount sufficient to specifically attenuate expression of the target genes, wherein one strand of

each double stranded RNA comprises a nucleotide sequence that is complementary to a region of at least 25 nucleotides of a target gene.

66. (New) A method for reducing or preventing an immune response in a recipient organism to a transplant tissue obtained from a donor organism, the method comprising supplying the transplant tissue with a double stranded RNA *in vitro* prior to implanting the transplant tissue into the recipient organism, wherein the double stranded RNA attenuates the expression of a target gene in the transplant tissue that can elicit an immune response in a recipient, and wherein one of the strands of the double stranded RNA is capable of hybridizing to the target gene *in vitro* in 400 mM NaCl, 40 mM PIPES pH 6.4, and 1 mM EDTA, at 50°C.

67. (New) A pharmaceutical composition for inhibiting the function of a target gene in a vertebrate cell, wherein the composition comprises a double stranded RNA in an amount sufficient to specifically attenuate expression of the target gene, wherein the double stranded RNA comprises a nucleotide sequence that is complementary to a region of at least 25 nucleotides of the target gene, and wherein the composition does not comprise phenol or chloroform.

68. (New) The method of any one of claims 48, 63, 64, 65 or 66 wherein the cell is a mammalian cell.

69. (New) The method of any one of claims 1, 48, 63, 64, 65 or 66 wherein the cell is a human cell.

70. (New) The method of any one of claims 23, 48, 63, 64, 65 or 66 wherein the cell is present in a mammal.

71. (New) The method of claim 70 wherein the cell is present in a human.

72. (New) The method of claim 63 or 65 wherein the target gene is associated with a disease.

73. (New) The method of claim 63 or 65 wherein the target gene is associated with a disease from a pathogen.

74. (New) The method of claim 1, 64, 65 or 66 wherein the double stranded RNA is supplied to the cell by delivering to the cell a DNA encoding the double stranded RNA.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Yin Xiong Li et al.	Art Unit:	1635
Serial No.:	10/038,984	Examiner:	Not Yet Assigned
Filed:	January 4, 2002	Customer No.:	
Title:	Composition and Method For *in Vivo* And *in Vitro* Attenuation of Gene Expression Using Double Stranded RNA		

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF YIN-XIONG Li, PH.D.

1. I am an inventor for the above-referenced patent application.

2. I have read the Office Action mailed July 31, 2001 in connection with parent application U.S.S.N. 09/493,301.

3. Non-specific effects reported by Oates *et al.* (Dev. Biol., 224:20-28, 2000) or Zhao *et al.* (Dev. Biol. 229:215-223, 2001), for example on page 220 of Zhao and page 24 of Oates, can be explained by differences between the methods used by Oates and Zhao and the methods taught in applicants' specification. For example, both Oates and Zhao use dsRNA that has been purified by phenol/chloroform extraction. In particular, phenol is toxic to zebrafish embryos and causes concentration-dependent, adverse effects in zebrafish. Thus, non-specific effects observed by Oates or Zhao are thought to be the result of toxic effects from residual phenol and/or chloroform in the dsRNA mixture

injected into the zebrafish embryos.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 08/08/02

Yin-Xiong Li, Ph.D.

P:\50238\50238.003002 Li Declaration.rtf

PATENT
Docket No. 275.00030102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Li et al.	Group Art Unit:	1635
Serial No.:	10/038,984	Examiner:	K. Lacourciere
Confirmation No.:	9705		
Filed:	4 January 2002		

For: COMPOSITION AND METHOD FOR IN VIVO AND IN VITRO ATTENUATION OF GENE EXPRESSION USING DOUBLE STRANDED RNA

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132
OF DR. YIN-XIONG LI

1. I am an inventor for the above-referenced patent application.

2. I received an MD with high honors from Hunan Medical University, China, in 1985 and received a Ph.D. in Molecular Biology and Biochemistry from Peking Union Medical College in 1991. In 1992 I was awarded the NIH Fogarty International Young Scientist Fellowship from the U.S. Department of Health and Human Services and came to the United States to pursue research focused on the molecular mechanism of heart development. I accepted a position in 2001 as Assistant Professor in the Departments of Pediatrics and Cell Biology at Duke University Medical Center. I have published 28 papers, an issued patent, and two patent applications related to my research.

3. This declaration is made in support of the amendment of the specification in the above-identified patent application at page 8, lines 3-4, at page 36, lines 11-12, and at Fig. 13, and further in support of the patentability of the pending claims.

4. The data recited in Example III (specification at page 36, line 6, bridging to page 37, line 7), and in Fig. 3 in the above-identified patent application as originally filed were

inadvertently incorrectly reported because the mammalian cells used were rat ROS cells, not mouse NIH/3T3 cells.

5. The specification is amended to recite rat cells in place of mouse NIH/3T3 cells or murine cells.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 04/22/03

Dr. Yin-Xiong Li. Ph.D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Yin Xiong et al.	Art Unit:	1635
Serial No.:	10/038,984	Examiner:	Not Yet Assigned
Filed:	January 4, 2002	Customer No.:	
Title:	Composition and Method For *in Vivo* And *in Vitro* Attenuation of Gene Expression Using Double Stranded RNA		

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 OF C. SATISHCHANDRAN, PH.D.

1. I have a Ph.D. degree in Biochemistry and Molecular Genetics. I am a professor at Jefferson Center for Biomedical Research and Chief Scientific Officer at Nucleonics, Inc., a company which focuses on double stranded RNA-mediated gene silencing research.

2. I have read the Office Action mailed July 31, 2001 in connection with parent application U.S.S.N. 09/493,301.

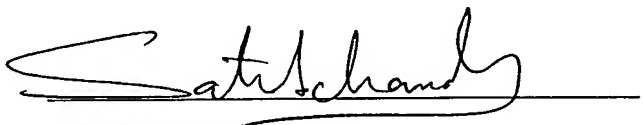
3. The attached post-filing reference (Ui-Tei *et al.*, FEBS Letters 479:79-82, 2000) demonstrates that dsRNA or a plasmid encoding dsRNA can be used to specifically silence a gene in mammalian Chinese hamster cells. This reference supports the ability of the presently claimed methods to specifically silence vertebrate (e.g., mammalian) genes.

4. A vector encoding dsRNA was used to specifically silence the prostate-specific antigen (PSA) gene in human rhabdomyosarcoma cells containing a PSA expression

plasmid (U.S.S.N. 10/062,707, filed January 31, 2002). Transfection of the vector encoding the dsRNA was mediated by lipofectamine (Gibco-BRL) according to the manufacturer's instructions. A greater than 95% reduction in PSA expression was seen in the cells expressing the PSA dsRNA. The inhibition was seen within two days after transfection and continued up until the last time point taken (one month later), at which point PSA levels were beginning to decline in the untreated cells and the experiment was terminated. The down-regulation was sequence specific: only the PSA derived dsRNA, and not the control HSV-2 derived dsRNA, induced down-regulation of PSA. Additionally, there was no toxicity associated with the cytoplasmic expression of the dsRNA.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 7/15/02



C. Satishchandran, Ph.D.

F:\50238\50238.003002 Satishchandran Declaration.rtf